JOURNAL OF VIROLOGY, Mar. 2008, p. 2652–2660 Vol. 82, No. 6
0022-538X/08/$08.00+0 doi:10.1128/JVI.02391-07

The DNA Deaminase Activity of Human APOBEC3G Is Required for Ty1, MusD, and Human Immunodeficiency Virus Type 1 Restriction▽

April J. Schumacher, Guylaine Haché, Donna A. MacDuff, William L. Brown, and Reuben S. Harris*

Department of Biochemistry, Molecular Biology and Biophysics, Institute for Molecular Virology, Beckman Center for Genome Engineering, University of Minnesota, Minneapolis, Minnesota 55455

Received 5 November 2007/Accepted 20 December 2007

Human APOBEC3G and several other APOBEC3 proteins have been shown to inhibit the replication of a variety of retrotransposons and retroviruses. All of these enzymes can deaminate cytosines within single-strand DNA, but the overall importance of this conserved activity in retroelement restriction has been questioned by reports of deaminase-independent mechanisms. Here, three distinct retroelements, a yeast retrotransposon, Ty1, a murine endogenous retrovirus, MusD, and a lentivirus, human immunodeficiency virus type 1 (HIV-1), were used to evaluate the relative contributions of deaminase-dependent and -independent mechanisms. Although human APOBEC3G can restrict the replication of all three of these retroelements, APOBEC3G lacking the catalytic glutamate (E259Q) was clearly defective. This phenotype was particularly clear in experiments with low levels of APOBEC3G expression. In contrast, purposeful overexpression of APOBEC3G-E259Q was able to cause modest to severe reductions in the replication of Ty1, MusD, and HIV-1(ΔVif). The importance of these observations was highlighted by data showing that CEM-SS T-cell lines expressing near-physiologic levels of APOBEC3G-E259Q failed to inhibit the replication of HIV-1(ΔVif), whereas similar levels of wild-type APOBEC3G fully suppressed virus infectivity. Despite the requirement for DNA deamination, uracil DNA glycosylase did not modulate APOBEC3G-dependent restriction of Ty1 or HIV-1(ΔVif), further supporting prior studies indicating that the major uracil excision repair system of cells is not involved. In conclusion, the absolute requirement for the catalytic glutamate of APOBEC3G in Ty1, MusD, and HIV-1 restriction strongly indicates that DNA cytosine deamination is an essential part of the mechanism.

The human apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G [A3G]) protein has been shown to inhibit the replication of a broad number of retroelements, including endogenous long-terminal-repeat retrotransposons such as Ty1 and MusD and exogenous retroviruses such as human immunodeficiency virus type 1 (HIV-1) (11–13, 18, 31, 34, 35, 42, 43, 54; reviewed in references 1, 6, 9, 20, and 21). This inhibition requires A3G to be incorporated into an assembling virus or virus-like particles through an RNA-dependent interaction with the retroelement's Gag protein (11, 26, 29, 33, 41, 49). A3G is then capable of deaminating cytosines to uracils in the first-strand cDNA during reverse transcription, which can result in hypermutation or degradation of the retroelement genome (Fig. 1) (see, e.g., references 2, 11–13, 18, 34, 35, 40, 42, 44, and 54).

A3G has two conserved zinc-binding domains, but only the carboxy-terminal (C-terminal) domain catalyzes the conversion of cytosine to uracil in single-strand DNA (5, 17, 39, 40). However, mutational analysis of the C-terminal zinc-binding domain suggested that A3G still had significant restriction activity in the absence of deaminase activity (11, 23, 39, 40, 44). The retrovirus restriction activity of A3G appeared to be significantly compromised only when both the amino-terminal (N-terminal) and C-terminal zinc-binding domains were mutated (11, 23, 40, 44). These results indicated that either domain is sufficient for A3G-mediated retroelement restriction, and they further suggested that DNA cytosine deamination per se might be dispensable (Fig. 1).

A possibly related but unresolved step in the mechanism of restriction is how A3G triggers the degradation of retroelement cDNA prior to integration (4, 8, 14, 16, 34, 35, 47, 50). Several groups have proposed that degradation of retroelement cDNA could be triggered by excision of A3G-dependent uracils by uracil DNA glycosylase (UDG) followed by abasic site cleavage (Fig. 1) (18, 20, 34, 35, 54). However, due to conflicting results, it is still unclear whether UDG has a role in A3G's restriction mechanism (25, 36, 53).

Our current studies test two main hypotheses: (i) A3G uses primarily a deaminase-dependent mechanism to inhibit retroelements, and (ii) cellular UDG contributes to A3G-mediated restriction by recognizing the resulting cDNA uracils. The first hypothesis was tested by titrating the expression of A3G or A3G zinc-binding site mutants in yeast and human cells and monitoring their ability to restrict a yeast retrotransposon, Ty1; a murine endogenous retroelement, MusD; and a lentivirus, HIV-1. The role of UDG was evaluated by monitoring A3G's ability to restrict Ty1 and HIV-1 in the absence of cellular uracil excision activity. The resulting data were consistent with a critical role for DNA cytosine deamination in Ty1, MusD, and HIV-1 restriction, but the main cellular UDG of yeast and humans appeared dispensable.

MATERIALS AND METHODS

Plasmids. Yeast constructs were based on pJG4-5 (Invitrogen). A3G was cloned with EcoRI/SalI from pTrc99A-A3G (17). A3G-E67Q was constructed by

* Corresponding author. Mailing address: Department of Biochemistry, Molecular Biology and Biophysics, Institute for Molecular Virology, Beckman Center for Genome Engineering, University of Minnesota, Minneapolis, MN 55455. Phone: (612) 624-0457. Fax: (612) 625 2163. E-mail: rsh@umn.edu.

▽ Published ahead of print on 9 January 2008.

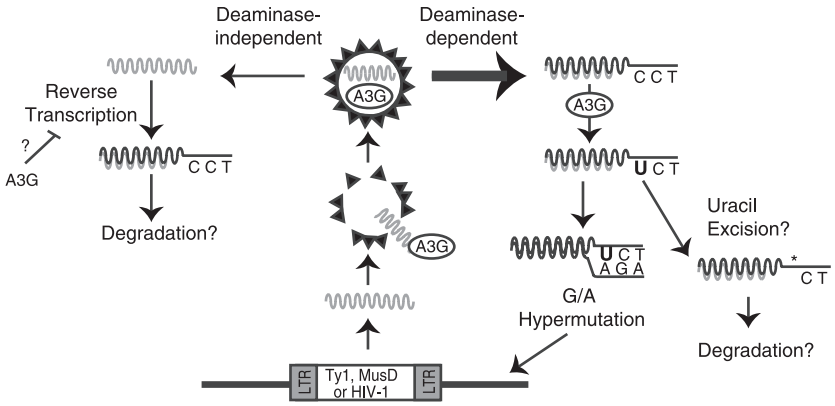

FIG. 1. Model depicting A3G deaminase-dependent and -independent retroelement restriction mechanisms. A3G is packaged along with the RNA genome (gray wavy lines) of a retroelement into assembling virus or virus-like particles. Following packaging, A3G uses either a deaminase-dependent or a deaminase-independent mechanism to inhibit replication at the reverse transcription step. The deaminase-dependent mechanism is depicted on the right. A3G deaminates cytosine to uracil in the first-strand cDNA (black wavy lines), which results in integration of an inactive retroelement due to G-to-A hypermutation or degradation, possibly as a result of uracil excision leaving an abasic site (*). The deaminase-independent mechanism is depicted on the left. A3G causes the degradation of the retroelement genome prior to integration, possibly by preventing reverse transcription. It should be noted that the Ty1, MusD, and HIV-1 life cycles have been oversimplified in order to focus on A3G's deaminase-dependent and -independent activities. Results from these studies indicate that A3G uses predominantly a deaminase-dependent restriction mechanism.

site-directed mutagenesis using primers 5′-TTA AGT ACC ACC CAC AGA TGA GAT TCT TC and 5′-GAA GAA TCT CAT CTG TGG GTG GTA CTT AA (Stratagene). A3G-E259Q was cloned with EcoRI/SalI from pTrc99A-A3G-E259Q (24). A3G-E67Q/E259Q was constructed by cloning the N-terminal domain of pJG4-5-A3G-E67Q by using EcoRI and RsrII into similarly cut pJG4-5-A3G-E259Q.

The A3G, A3G-E67Q, and A3G-E259Q pcDNA3.1 eukaryotic expression plasmids used in the HIV-1 single-cycle assay have been described previously (17, 32). A3G-E67Q/E259Q was cloned with KpnI/SalI from pTrc99A-A3G-E67Q/E259Q into similarly cut pcDNA3.1. pTrc99A-A3G-E67Q/E259Q was constructed by cloning the N-terminal domain of pTrc99A-A3G-E67Q using KpnI and BamHI into similarly cut pTrc99A-A3G-E259Q.

The A3G expression constructs used in the MusD studies were created by amplifying wild-type A3G and mutant derivatives from pcDNA3.1 expression plasmids using oligonucleotides 5′-NNN NGA GCT CAG GTA CCA CCA TGA AGC CTC ACT TCA GAA AC and 5′-NNN GTC GAC TCC GTT TTC CTG ATT CTG GAG AAT and were cloned using KpnI/SalI into pcDNA3.1-HA cut with KpnI/XhoI (48). Wild-type hemagglutinin-labeled A3G (A3G-HA) and the A3G-HA mutants were cloned using HindIII/XbaI into similarly cut pEAK8-KS+ (19). Enhanced green fluorescent protein (EGFP) was cloned using NheI/NotI from pEGFP-N3 (Clontech) into pEAK8-KS+ cut with XbaI/NotI. The pEAK8-KS+ constructs were used for the MusD assay.

Full-length infectious HIV-1 (with or without Vif) molecular clones were kindly provided by Michael Malim (King's College London, London, England) (46). The MusD expression plasmid was a gift from Thierry Hiedmann (Institute Gustave Roussy, Villejuif, France) (12).

Yeast strains. Yeast mutation assays and endogenous retrotransposition assays were performed with *Saccharomyces cerevisiae* strains DG1141 (*matα trp1-hisG ura3-167 his3Δ200* Ty1-2y2his3AI) or DG1141 ΔUNG1 (*matα trp1-hisG ura3-167 his3Δ200* Ty1-2y2his3AI *ung1*::*kanMX4*) (42). DG1141 ΔUNG1 was constructed by amplifying the *ung1*::*kanMX4* cassette from yeast deletion strain 36067 (R. Wright, University of Minnesota), transforming DG1141 with the resulting PCR product, and selecting G418-resistant colonies (51). The Ung1 deletion was confirmed by insert-specific PCRs and by screening for a modest CAN1 mutator phenotype (as described previously [42]).

Cell culture and generation of stable clones. The CEM-GFP (AIDS Reference and Reagent Program), CEM, and CEM-SS (Michael Malim, King's College London, London, England) T-cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bioproducts). HeLa cells and 293T cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum. Growth media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Stable CEM-SS clones expressing A3G, A3G-E67Q, A3G-E259Q, or A3G-E67Q/E259Q were generated by electroporation-mediated transfection of CEM-SS cells with 30 μg of linearized plasmid, followed by serial dilution in 96-well plates and selection in medium containing 1 mg/ml G418 (Mediatech, Inc.). After approximately 14 days of incubation, clones were picked, expanded, and maintained in medium containing 0.5 mg/ml G418. We successfully generated stable cell lines expressing wild-type A3G, A3G-E67Q, and A3G-E259Q at near-physiologic levels; however, we were able to generate A3G-E67Q/E259Q cell lines only with expression levels slightly lower than those of the other A3G derivatives.

Endogenous Ty1 retrotransposition and yeast mutation assays. pJG4-5 and its derivatives were transformed into DG1141 or DG1141 ΔUNG1 and selected using a synthetic complete medium lacking tryptophan (SC-TRP). Individual colonies were used to inoculate 1 ml of SC-TRP plus 3% raffinose (RAF) and were grown to saturation at 30°C. Five thousand to 10,000 cells were transferred to 2 to 3 ml of SC-TRP plus 3% galactose (induced expression experiments) or SC-TRP plus 3% RAF (noninduced expression experiments) and were grown to saturation at 20°C for 7 to 10 days. Cell viability was determined by plating a dilution on rich medium. Retrotransposition and mutation frequencies were determined by plating cultures on SC lacking histidine or SC lacking arginine plus canavanine, respectively (described in reference 42).

MusD transposition assay. HeLa cells were plated in 6 well plates at 1×10^5 cells per well. Twenty-four hours later, cells were transfected with 0.6 μg MusD plasmid, 0.3 μg EGFP expression plasmid, and various amounts (0.06 to 0.6 μg) of the human A3G expression plasmid and/or empty vector using FuGene 6 (Roche)-mediated transfection (1.5 μg total DNA/transfection). Two days post-transfection, 10% of the cells were analyzed for EGFP expression by flow cytometry and the rest plated into 100-mm dishes in the presence of 800 μg/ml G418. After 14 days, G418-resistant colonies were fixed, stained with crystal violet solution, and counted (12, 13).

Single-cycle HIV infectivity assays and spreading infections. For the single-cycle infectivity assays, HIV-GFP virus stocks were produced by FuGene 6-mediated transfection of 50 to 70% confluent 293T cells with the following plasmid cocktail: 0.22 μg CS-CG, 0.14 μg pRK5/Pack1(Gag-Pol), 0.07 μg pRK5/Rev, 0.07 μg pMDG (vesicular stomatitis virus G protein), along with 0.02, 0.1, or 0.5 μg of an APOBEC3G expression or empty-vector control plasmid (32, 37). An empty-vector control was used to adjust all transfections to 1 μg. After 48 h of incubation, virus-containing supernatants were filtered (pore size, 0.45 μm) and used to infect fresh 293T target cells. Virus-producing 293T cells were also harvested and used to quantify transfection efficiency by quantifying GFP-positive cells by flow cytometry (FACSCalibur; Becton Dickinson). After an additional 48 to 72 h of incubation, the transduced 293T target cells were harvested and used to quantify infectivity by flow cytometry.

For the spreading infections, virus stocks were produced by FuGene 6-mediated transfection of 5 μg of HIV-1 provirus plasmid into 293T cells (10-cm dish, 50 to 70% confluent). After 48 h of incubation, virus-containing supernatants were filtered (pore size, 0.45 μm) and titered using a CEM-GFP reporter system

(15). A multiplicity of infection of 0.05 was used to initiate spreading infections. Virus infectivity was monitored periodically by mixing 25,000 CEM-GFP cells with 125 μl of cell-free culture supernatant. After incubation for 48 h, CEM-GFP cells were fixed with 4% paraformaldehyde, and infectivity levels (GFP expression) were monitored by flow cytometry.

Immunoblotting. Yeast whole-cell extracts were obtained from cultures conferring the median frequency of retrotransposition during endogenous Ty1 retrotransposition assays by subjecting the pellets to trichloroacetic acid (TCA) precipitation (see, e.g., reference 42). HeLa whole-cell extracts were prepared from confluent 6-well plates. Cells were trypsinized, washed in phosphate-buffered saline, and lysed in 35 μl lysis buffer (25 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 10% glycerol, 1% NP-40, and Complete protease inhibitors [Roche]). Lysates were incubated on ice for 1 h and spun at 21,000 × *g* for 10 min, and the supernatants were added to 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. 293T or CEM-SS whole-cell extracts were prepared from confluent 6-well plates using a standard freeze-thaw protocol. For the virion incorporation assays, viral particles were purified by centrifugation (16,000 × *g*, 2 h) through a 20% sucrose cushion. Protein or viral particle samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with antibodies to HA (Covance), α-tubulin (Covance), PSTAIRE (Cdc28; gift from Duncan Clarke), A3G, or p24 (40, 45).

UDG activity assays. Yeast whole-cell extracts were prepared by growing DG1141 or DG1141 ΔUNG1 to an optical density at 600 nm of 0.6 in 50 ml rich medium. Cells were pelleted and washed twice with Tris-buffered saline. Cells were resuspended in 400 μl 50 mM HEPES (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 0.2 mM pepstatin, and 0.4 mM benzamidine and were then lysed by vortexing with 200 μl glass beads. CEM-SS-A3G or CEM-SS-A3G-plus-Ugi whole-cell lysates were prepared using HED buffer (25 mM HEPES, 5 mM EDTA, 1 mM dithiothreitol, 10% glycerol [pH 7.8] containing Complete protease inhibitor [Roche]). Yeast and T-cell whole-cell extracts were analyzed for UDG activity as described previously (10). Whole-cell extracts were incubated with 1 pmol of a fluorescein isothiocyanate (FITC)-labeled deoxyoligonucleotide substrate containing a single uracil base at position 16 (5′-ATT ATT ATT ATT CCG UGG ATT TAT TTA TTT ATT TAT TTA TTT-FITC) in a final volume of 10 μl at 37°C for 2 h. The reaction was terminated by the addition of 10 μl of formamide loading dye. The products were resolved on 15% Tris-borate-EDTA-urea polyacrylamide gels and were visualized using a FLA-5000 phosphorimager (Fuji). The existing apurinic-apyrimidinic endonuclease activity present in the extracts was sufficient to cleave all the deglycosylated substrate generated during the reaction. As controls, the lysate was replaced with 0.1 U of recombinant UDG (Roche) or preincubated with 2 U of UDG inhibitor (Ugi; New England Biolabs) for 10 min on ice before addition of the oligonucleotide substrate.

RESULTS

A3G N- or C-terminal zinc-binding domain mutants mediate Ty1 restriction at induced expression levels. To begin to address the relative contributions of deaminase-dependent or -independent mechanisms, we tested whether A3G N- or C-terminal zinc-binding domain mutants would be able to restrict the transposition of a yeast endogenous retroelement, Ty1. A3G single and double zinc-binding site mutants were created by changing the conserved glutamate (E) in each zinc-binding domain to glutamine (Q). Each A3G mutant was expressed in yeast from a plasmid with a galactose-inducible promoter, and the abilities of these mutants to inhibit Ty1 retrotransposition was compared to that of wild-type A3G. The N- and C-terminal zinc-binding domain mutants A3G-E67Q and A3G-E259Q, respectively, inhibited Ty1 retrotransposition by 60 to 70% (Fig. 2A). These decreases were similar to those caused by wild-type A3G (80% [42]). In contrast, the double zinc-binding domain mutant A3G-E67Q/E259Q did not significantly inhibit transposition. These results roughly paralleled previously reported data on galactose-induced A3G expression (11). Overall, these data indicated that under high-expression conditions (3% galactose), either zinc-binding domain of A3G could mediate Ty1 restriction (i.e., the domains appear to have redundant function).

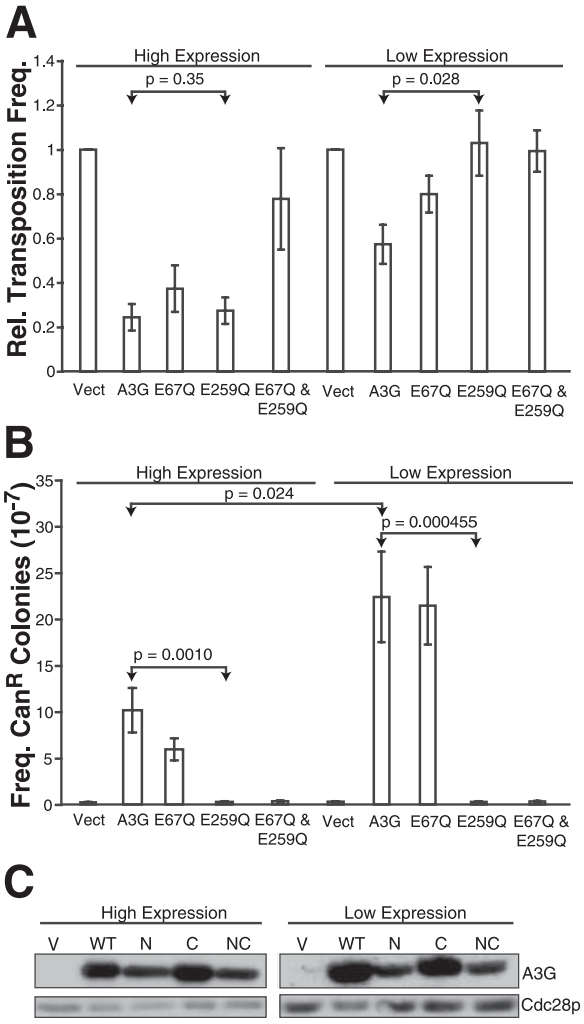

FIG. 2. Ty1 restriction by A3G and A3G zinc-binding domain mutants. (A) Effect of A3G or A3G mutants on Ty1 retrotransposition under high and low expression conditions as monitored by the frequency of His^+ colonies. For each condition, the averaged median frequency of Ty1 retrotransposition from five independent experiments, each performed with 8 to 16 independent yeast cultures, was normalized to the vector controls, which had an average frequency of Ty1 retrotransposition of 160×10^{-8}. Error bars, 1 standard errors of the means. *P* values for the indicated data sets were calculated using the *t* test. (B) Yeast mutation assay monitoring the frequency of canavanine-resistant (Can^R) colonies for yeast expressing wild-type A3G or A3G mutants for six independent experiments, each performed with 8 to 16 independent cultures. The averaged median mutation frequencies and the corresponding standard errors of the means are shown. *P* values for the indicated data sets were calculated using the *t* test. (C) Immunoblots of TCA protein extracts from yeast cells expressing a vector control (V), B42-A3G (wild type [WT]), B42-A3G E67Q (N-terminal mutant [N]), B42-A3G E259Q (C-terminal mutant [C]), or B42-A3G E67Q/E259Q (double mutant incorporating both N- and C-terminal mutations [NC]). Cdc28p is a loading control. It should be noted that five times more cell lysate was loaded for the low-expression immunoblots and A3G could be detected only after an overnight exposure.

To confirm that E259Q (but not E67Q) abolished the

deaminase activity of A3G, all of the constructs were tested for deaminase activity using a canavanine resistance (Can^R) yeast mutation assay (42). As expected based on previous studies, A3G-E67Q was deaminase proficient, triggering a mutation frequency increase resembling that of wild-type A3G (Fig. 2B). In contrast, A3G-E259Q and A3G-E67Q/E259Q were clearly deaminase deficient. The Can^R mutation data confirmed that A3G-E259Q has no deaminase activity in yeast and, together with the Ty1 data, demonstrated that the decrease in Ty1 retrotransposition caused by A3G-E259Q at high expression levels is deaminase independent.

We next asked whether any of the retrotransposition phenotypes were due to differential expression levels. Expression of A3G and each of the A3G mutants was assayed by immunoblotting. Wild-type A3G and A3G-E259Q were expressed similarly, but A3G-E67Q and A3G-E67Q/E259Q appeared to be expressed at levels approximately two- to threefold lower (Fig. 2C, left panel). Therefore, it was possible that the modest recovery of retrotransposition frequency due to E67Q might actually be due to a defect in expression or protein stability.

Mutation of the C-terminal zinc-binding domain eliminates A3G-mediated restriction of Ty1 under noninduced conditions. We were concerned that Ty1 restriction by the A3G zinc binding-domain mutants could be an artifact of the high-expression conditions. Since the galactose promoter is leaky in the absence of glucose, we tested whether A3G and the A3G zinc-binding domain mutants could inhibit Ty1 retrotransposition under noninducing conditions. Interestingly, at low expression levels (0% galactose and 3% RAF), only wild-type A3G and A3G-E67Q significantly inhibited Ty1 retrotransposition (45% and 23%, respectively), whereas A3G-E259Q and A3G-E67Q/E259Q allowed levels of Ty1 retrotransposition similar to that with the vector control (Fig. 2A). The deaminase activities of A3G and each of the A3G zinc-binding site mutants under low-expression conditions were compared to the deaminase activities observed at high expression. As expected, the two A3G variants containing the E259Q mutation showed frequencies of Can^R colonies indistinguishable from that with the vector control. Moreover, under low-expression conditions, A3G or A3G-E67Q caused mutation frequencies that were 2.2- and 3.6-fold higher, respectively, than those under high-expression conditions (Fig. 2B). The clear disconnect between expression levels and mutability suggested that high A3G expression levels might be somewhat artifactual (see Discussion).

The expression levels of A3G and its derivatives under noninducing conditions were monitored by immunoblotting. As observed in the fully induced expression experiments, A3G and A3G-E259Q were expressed similarly and the A3G-E67Q mutant (or the double mutant) was expressed at a lower level (Fig. 2C, right panel). The fact that the high and low expression data mirror one another suggests that E67Q compromises A3G stability. Reconstruction experiments showed that inducing A3G expression in yeast resulted in approximately 100-fold more protein than that under noninducing conditions (data not shown). Therefore, in contrast to the results of the assays conducted under high-expression conditions, these results indicate that the C-terminal glutamate (E259), and thus DNA deamination, is required for A3G-mediated restriction of Ty1.

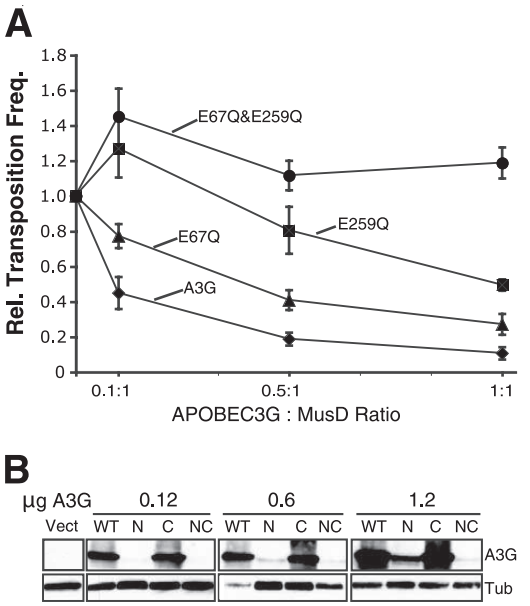

FIG. 3. MusD restriction by A3G and zinc-binding domain mutants. (A) Effects of varying amounts of A3G or mutant derivatives on MusD transposition relative to that with the vector control. Transposition was monitored by the number of G418-resistant colonies. The means and standard errors of the means for four independent experiments are shown; each individual experiment used three cultures per condition. Rel., relative; Freq., frequency. (B) Representative immunoblot of lysates from HeLa cells expressing a vector control (Vect), A3G-HA (wild type [WT]), A3G-E67Q-HA (N-terminal mutant), A3G-E259Q-HA (C-terminal mutant), or A3G-E67Q/E259Q-HA (double mutant with both N- and C-terminal mutations). Tubulin (Tub) is a loading control. Amounts of A3G correspond to ratios as follows: 0.12 μg, 0.1:1 ratio; 0.6 μg, 0.5:1 ratio; 1.2 μg, 1:1 ratio. All of the samples were run on the same gel and cropped to be in the same order as the ratios in panel A.

Mutation of the C-terminal zinc-binding domain compromises A3G-mediated restriction of MusD in a dose-dependent manner. A3G has also been shown to inhibit a murine endogenous retroelement, MusD (12, 13). However, whether A3G inhibits MusD through a deaminase-dependent or a deaminase-independent mechanism has not been tested. To test whether the catalytic glutamate of A3G (E259) is required for restriction of a mammalian endogenous retroelement, we transfected varying amounts of A3G and mutant derivatives into HeLa cells and monitored their abilities to inhibit MusD transposition. At a 1:1 ratio of A3G to MusD, wild-type A3G and the N- and C-terminal zinc-binding domain mutants caused significant decreases in MusD transposition (89%, 73%, and 51%, respectively). However, at 0.5:1 or 0.1:1 ratios, only wild-type A3G (81% or 55%, respectively) and A3G-E67Q (59% or 33%, respectively) showed significant decreases in MusD transposition, whereas A3G-E259Q clearly did not (Fig. 3A). As expected, the double zinc-binding domain mutant failed to inhibit MusD transposition under any of the expression conditions tested. We also noted that low levels of A3G-E259Q or the double mutant caused a reproducible increase in MusD transposition frequencies. We do not have a molecular explanation for this effect, but it is formally possible that transposition may be stimulated by low protein levels

(regardless of E67Q or E259Q) and that any increase is readily overcome by catalytically active protein.

Wild-type A3G and A3G-E259Q were similarly detectable by immunoblotting under all of the expression conditions (Fig. 3B). However, due to the decreased stability of mutants containing the E67Q mutation, A3G-E67Q and A3G-E67Q/E259Q could not be compared, because they could not be detected at the 0.1:1 ratio. Therefore, we doubled the amount of the A3G expression construct transfected into HeLa cells (1.2 μg, 0.6 μg, and 0.12 μg) and repeated the Western blotting for each expression condition. Even under these conditions, A3G-E67Q was almost undetectable at the lowest expression level and A3G-E67Q/E259Q was still undetectable (Fig. 3B). It is noteworthy that, despite expression of undetectable levels of A3G-E67Q (0.1:1 ratio), significant decreases in MusD transposition were still observed. These data indicate that even extremely low levels of A3G-E67Q expression are sufficient to mediate restriction, and they are further consistent with a requirement for E259 and DNA deamination in MusD restriction.

Mutation of the C-terminal zinc-binding domain eliminates A3G-mediated restriction of HIV-1(ΔVif) in a dose-dependent manner. To test if A3G-mediated restriction of HIV-1(ΔVif) occurs by a deaminase-dependent or -independent mechanism, we transfected varying amounts of A3G or the A3G zinc-binding mutants into 293T cells and monitored their abilities to inhibit HIV-1(ΔVif) in a single-cycle infectivity assay (Fig. 4A) (17, 24, 32). Under conditions where maximal levels of A3G were used (a 2:1 ratio of A3G to HIV-1), A3G-E67Q and A3G-E259Q inhibited HIV-1(ΔVif) infectivity almost as efficiently as wild-type A3G (94% and 95%, respectively). At a ratio of 0.5:1, A3G-E67Q and A3G-E259Q showed reduced but similar restriction activity against HIV-1(ΔVif) (77% and 62%, respectively). However, at a ratio of 0.1:1, only wild-type A3G and A3G-E67Q significantly inhibited HIV-1(ΔVif) infectivity (91% and 46%, respectively [Fig. 4A]).

To confirm that loss of restriction was not due to a decrease in A3G protein expression or virion encapsidation, we performed immunoblotting on cell lysates and virus particles. A3G-E259Q was expressed in cells at levels similar to those of wild-type A3G, but decreases were again observed for A3G-E67Q and the double mutant (Fig. 4B). Despite detection of A3G-E67Q and A3G-E67Q/E259Q in cells, only the highest expression conditions enabled A3G-E67Q to be detected in virus particles (Fig. 4B). A3G-E67Q/E259Q encapsidation was not detected. The lower levels of virion-associated A3G-E67Q suggested that this mutation interferes with efficient A3G packaging. Other studies have indicated that this defect is most likely due to reduced nucleic acid binding activity (23, 39; also unpublished data).

Despite a packaging defect, A3G-E67Q still caused significant decreases in HIV-1(ΔVif) infectivity (Fig. 4A). These data suggested that, once encapsidated, A3G-E67Q can effectively mediate efficient restriction. These results echoed the MusD restriction data, where undetectable levels of A3G-E67Q were inhibitory. Furthermore, when the expression and packaging defects of A3G-E67Q were taken into account, this protein appeared to restrict as well as wild-type A3G. For instance, when similar amounts of protein were packaged, these two proteins showed nearly identical decreases in HIV-1(ΔVif) infectivity (94% and 91%, respectively; ratio of A3G-E67Q to wild-type A3G, 2 to 0.1) (Fig. 4C, 2nd and 10th bars). In contrast, A3G-E259Q did not inhibit HIV-1(ΔVif) infectivity when similarly expressed and encapsidated (Fig. 4C, fifth bar), again indicating the importance of DNA cytosine deamination activity.

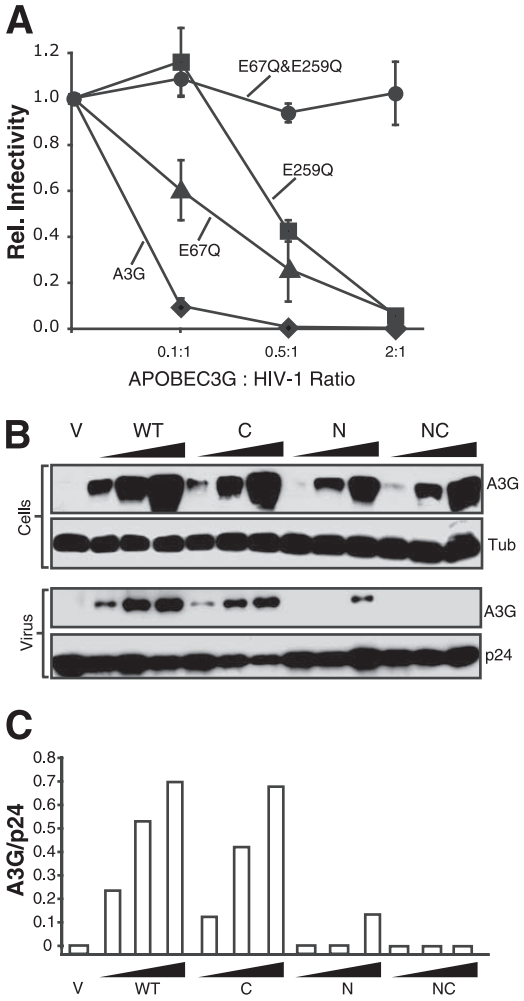

FIG. 4. HIV-1 restriction by A3G and A3G zinc-binding domain mutants. (A) Results of a representative HIV-1(ΔVif) single-cycle infectivity assay from four independent experiments, monitoring the effects of different amounts of A3G or A3G mutants on HIV-1(ΔVif) infectivity. Each data point represents the average of two independent cultures relative to the vector control. The standard deviation between the two data sets is shown (error bars); where not visible, it is smaller than the data point. Rel., relative. (B) Immunoblot of lysates from 293T cells (top) expressing a vector control (V), A3G (wild type [WT]), A3G-E67Q (N-terminal mutant), A3G-E259Q (C-terminal mutant), or A3G-E67Q/E259Q (double mutant with both N- and C-terminal mutations) or virion encapsidation of A3G and its variants into viral particles (bottom). Tubulin (Tub) and p24 are loading controls. (C) Quantification of the amounts of A3G and its variants incorporated into viral particles from panel B.

Mutation of the C-terminal zinc-binding domain eliminates A3G-mediated restriction of HIV-1 in a spreading infection. To study the importance of the N- and C-terminal zinc-binding domains in HIV-1 restriction under more physiological conditions, we constructed CEM-SS T cell lines stably expressing

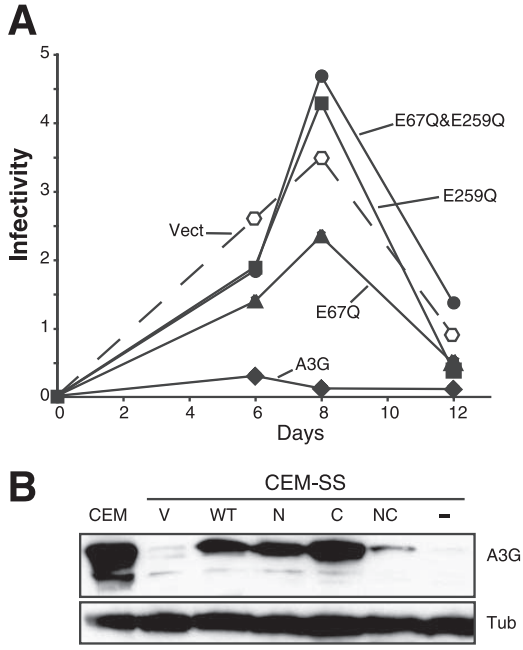

FIG. 5. Effect of A3G or A3G zinc-binding domain mutants on spreading HIV-1(ΔVif) infections. (A) Representative data showing the replication of HIV-1(ΔVif) on CEM-SS cells stably expressing a vector control (Vect), wild-type A3G, or mutant A3G. (B) Immunoblot of lysates from untransfected CEM-SS cells (−) or CEM-SS cells stably expressing a vector control (V), A3G (wild type [WT]), A3G-E67Q (N-terminal mutant), A3G-E259Q (C-terminal mutant), or A3G-E67Q/E259Q (double mutant with both N- and C-terminal mutations). Tubulin (Tub) is a loading control. CEM cells were used as a control for nonpermissive A3G expression levels.

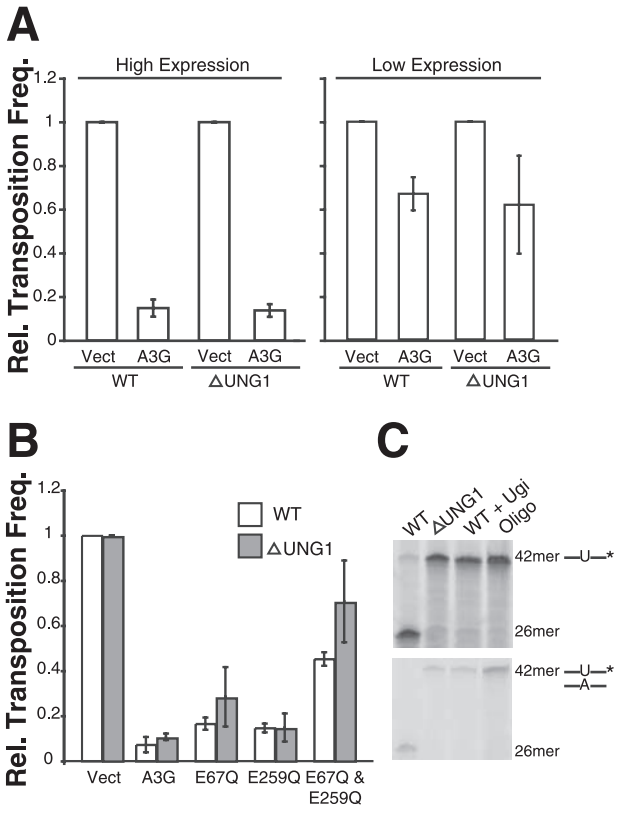

FIG. 6. A3G-dependent restriction of Ty1 is independent of Ung1p. (A) Restriction of Ty1 by wild-type A3G. Results are from two independent experiments performed under inducing or noninducing conditions in the presence (WT) or absence (ΔUNG1) of Ung1p. Histograms show the averaged median retrotransposition frequencies (Freq.) relative (Rel.) to those of the vector control (Vect), which had average Ty1 retrotransposition frequencies of 130×10^{-8} and 110×10^{-8} for the high- and low-expression experiments, respectively. Error bars, standard errors of the means. (B) Effects of A3G zinc-binding domain mutants on Ty1 retrotransposition in the presence and absence of Ung1p. Histograms show the normalized averaged medians and standard errors of the means from two independent experiments. (C) Assay for uracil DNA glycosylase activity on yeast cell lysates from the wild-type (WT) and Ung1p deletion (ΔUNG1) strains. Lysates were incubated with a fluorescein-labeled 42-mer single stranded (top) or double stranded (bottom) oligonucleotide. A cleaved oligonucleotide results in a 26-mer. The last two lanes show the effects of recombinant Ugi on the wild-type strain and the oligonucleotide alone (Oligo), respectively.

A3G and each of the A3G zinc-binding domain mutants at, or slightly below, physiological levels (Fig. 5B). It should be stressed that the levels of A3G and derivative proteins expressed in these experiments were comparable to those in T-cell lines (CEM, H9) and in human peripheral blood mononuclear cells (Fig. 5) (G. Haché et al., unpublished data). A3G- or mutant derivative-expressing CEM-SS clones were infected with replication-proficient HIV-1(ΔVif) and monitored over a 2-week period.

As observed at lower expression levels in single-cycle retrotransposon and HIV-1 restriction assays, A3G-E259Q was unable to restrict the spreading replication of HIV-1(ΔVif) (Fig. 5A). Again, A3G-E67Q showed intermediate levels of restriction, but this can be attributed to an encapsidation defect (Fig. 4B). The inability of catalytically inert A3G-E259Q to inhibit the replication of HIV-1(ΔVif) at near-physiological expression levels demonstrates the importance of DNA cytosine deamination in A3G-mediated restriction.

UDG does not contribute to A3G-mediated restriction of Ty1. To gain further insight into A3G's deaminase-dependent mechanism, we asked if UDG contributed to the degradation of deaminated retroelements. *S. cerevisiae* provided an ideal system with which to address this question, because it has only one UDG, called uracil DNA *N*-glycosylase 1 protein (Ung1p), unlike most other eukaryotes, including humans, which have multiple UDGs (30).

We used homologous recombination to construct an Ung1p deletion strain (DG1141 *ung1*::*kanMX4*) to test whether uracil excision contributed to A3G-dependent restriction. A yeast mutation assay confirmed the *UNG1* deletion: this strain showed a slight increase in the frequency of spontaneous mutation (Can^R colonies) in comparison to the wild-type strain (data not shown) (42). Moreover, the Ung1p deletion strain had no detectable uracil excision activity, as determined by cleavage of uracil-containing single- or double-strand DNA oligonucleotides (Fig. 6C).

The retrotransposition frequency of Ty1 was assayed in wild-type and Ung1p deletion strains in the presence and absence of A3G. Under high expression levels (3% galactose), A3G inhibited Ty1 retrotransposition by 90% in the presence or absence of Ung1p. To eliminate the nonspecific restriction effects

of the N-terminal domain at high expression levels, we also tested each of the A3G zinc-binding domain mutants for Ty1 restriction in the presence and absence of Ung1p. Like that by wild-type A3G, Ty1 restriction by A3G-E67Q, A3G-E259Q, or A3G-E67Q/E259Q was not significantly different in the absence of Ung1p (Fig. 6B).

To further confirm that Ung1p has no effect on A3G-mediated restriction of Ty1, the assay was repeated using noninducing conditions (0% galactose, 3% RAF). No significant differences in Ty1 restriction were seen in the presence or absence of Ung1p when A3G was expressed minimally (26% and 28%, respectively) (Fig. 6A). The high- and low-expression data therefore combine to show that, despite the requirement for the catalytic glutamate in Ty1 restriction and the likely accumulation of cDNA uracils, Ung1p does not appear to contribute to A3G-dependent restriction.

Inhibition of UNG2 has no effect on A3G-mediated restriction of HIV-1. To test if UDG plays a role in A3G-mediated restriction of HIV-1(ΔVif), we expressed a UDG inhibitor (Ugi) in CEM-SS cells stably expressing near-physiologic levels of A3G (Fig. 5B). Ugi potently blocks the uracil excision activity of human UNG2 but not that of minor cellular UDGs such as SMUG1 (30). UDG activity was monitored in cells stably expressing Ugi by incubating cell extracts with a double-strand florescent DNA oligonucleotide containing a single uracil. CEM-SS cells expressing Ugi had no detectable uracil excision activity (Fig. 7C). We then infected the cell lines with replication-competent HIV-1 or HIV-1(ΔVif) and monitored A3G-mediated restriction of virus replication. Consistent with previously published results, Ugi did not affect virus replication or suppress A3G-mediated restriction of HIV-1(ΔVif) (Fig. 7A and B) (25). These results supported the Ty1 studies and indicated that A3G-dependent restriction of HIV-1(ΔVif) is not influenced by the main cellular UDG of humans, UNG2.

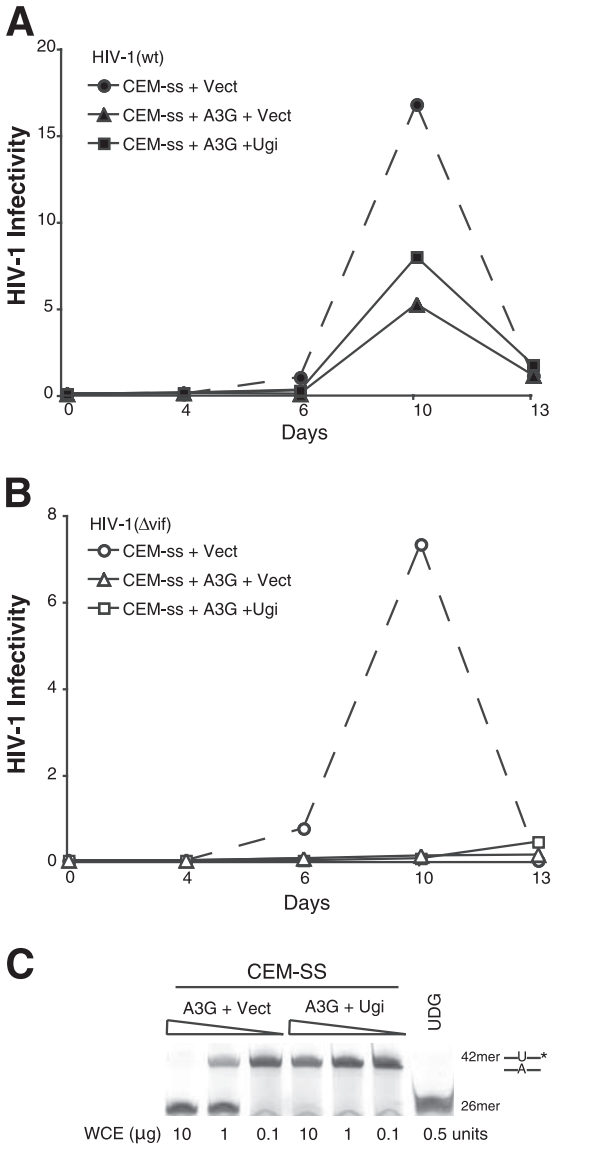

FIG. 7. A3G-dependent restriction of HIV-1 is independent of UNG. (A and B) Effect of wild-type A3G on infection with wild-type (wt) HIV-1 (A) or HIV-1(ΔVif) (B) in the presence or absence of uracil excision activity. Vect, vector. (C) Oligonucleotide cleavage assay for uracil DNA glycosylase activity on CEM-SS whole-cell extracts (WCE). Lysates were incubated with a fluorescein-labeled 42-mer double-stranded oligonucleotide. Cleavage results in a 26-mer. The last lane shows the effect of recombinant UDG.

DISCUSSION

These studies have addressed whether A3G uses predominately a deaminase-dependent or a deaminase-independent mechanism to inhibit the replication of a yeast retrotransposon, Ty1, a murine endogenous retrovirus, MusD, and a lentivirus, HIV-1. Minimal expression of a deaminase-defective mutant (A3G-E259Q) abolished A3G-mediated restriction of all three retroelements, while an equivalent mutation in the pseudoactive N-terminal zinc-binding domain (A3G-E67Q) did not. These results indicated that the antiretroelement activity of A3G is intrinsic to the protein, since restriction was dependent on E259 in three different systems. A3G-E259Q is virtually indistinguishable from wild-type A3G in terms of its cellular expression level, encapsidation ability, cellular localization, and dimerization capacity (these studies and unpublished data). Therefore, we strongly favor the conclusion that the deaminase activity of A3G is absolutely crucial for inhibiting the replication of Ty1, MusD, and HIV-1(ΔVif). Despite this requirement, the major uracil excision enzymes of yeast and humans did not appear to influence A3G-mediated retroelement restriction.

Our studies suggest that much of the previous data on A3G's deaminase-independent activity can be explained by expression differences. Consistent with previously published results, overexpression of the zinc-binding domain mutants A3G-E67Q and A3G-E259Q caused decreases in HIV-1(ΔVif) infectivity similar to those with wild-type A3G (23, 39, 40, 44). However, when A3G was expressed minimally, mutation of the catalytic glutamate (E259Q) eliminated A3G-mediated restriction. Furthermore, stable T-cell lines expressing A3G-E259Q at near-physiologic levels failed to inhibit spreading infections of HIV-1(ΔVif).

We previously demonstrated that A3G can inhibit Ty1 and mutate yeast genomic DNA (42). The present study extends this work by showing that the mechanism of Ty1 restriction parallels those of MusD and HIV-1(ΔVif) restriction. This was

particularly apparent at minimal expression levels. Together with the data revealing that lower levels of A3G expression actually triggered higher Can^R mutation frequencies, we hypothesize that higher expression levels (particularly in heterologous systems) may lead to A3G protein aggregation, to less functional protein (albeit more overall), and possibly to artifactual observations. For instance, one can easily envisage that the overexpression of A3G could lead to a higher-molecular-weight aggresome, which could immobilize the genomic RNA and/or Gag protein of assembling retroelements (see, e.g., references 7, 28, and 52). Such an explanation readily accounts for Ty1 and HIV-1 data reported here and in previous publications (11, 39, 40, 44).

However, because A3G-E67Q does not inhibit Ty1, MusD, or HIV-1(ΔVif) as efficiently as wild-type A3G, we cannot completely eliminate the possibility that the N-terminal zinc-binding domain contributes some sort of deaminase-independent activity. Nevertheless, it should be noted that this possibility is unlikely, because the weak activity of A3G-E67Q can be attributed to lower expression levels and/or diminished encapsidation abilities. Our results are consistent with a report by Mbisa and colleagues, which showed that the catalytic activity of A3G is required for the inhibition of HIV-1 plus-strand transfer and integration (36).

Since DNA cytosine deamination is critical for A3G-mediated restriction of Ty1, MusD, and HIV-1(ΔVif), the apparent lack of involvement of cellular UDGs was surprising. Unlike a recent report, which showed that UNG2 is necessary for efficient HIV-1 replication and A3G-mediated restriction of HIV-1(ΔVif), we showed that UNG2 had no effect on HIV-1 replication or on the ability of A3G to inhibit HIV-1(ΔVif) (53). Furthermore, we showed that deletion of the only uracil excision enzyme in yeast (Ung1p) had no effect on A3G-mediated restriction of Ty1. The reasons for these discrepancies are not clear. However, our HIV-1 results are more consistent with two other recent reports, which showed that UNG2 has no effect on virus infectivity or on the accumulation of viral DNA in the presence of A3G (25, 36). One of these studies also showed that another cellular UDG (SMUG1), whose activity was not detected here in CEM-SS T cells, was not involved (25). Moreover, our present data also addressed the UNG2 hypothesis without potential contributions from A3F (virus replication experiments using A3F- and A3G-expressing H9 cells [25]) or complications from A3G overexpression (transient-transfection single-cycle infectivity experiments [36]), thereby ruling out the possibility of the virus simply being too burdened with hypermutations to show infectivity recoveries in the absence of UNG2. Nevertheless, despite the strong requirement for the DNA deamination activity of A3G, the main uracil excision enzymes of yeast and humans appear dispensable.

Finally, it should be stressed that our data may not extend directly to other APOBEC3 proteins or other systems. Here we address only human A3G and Ty1, MusD, and HIV-1, but our data can certainly provide useful points for comparison. Similar experiments must be done with other systems (ideally with at least one reference element; such as HIV-1), because it is feasible that deaminase-independent mechanisms do indeed exist. For instance, data from several groups concur that the restriction of the endogenous retrotransposons L1 and Alu occurs by a deamination-independent mechanism (3, 22, 27, 38, 48). We further recognize that future experiments must also endeavor to address each APOBEC3–retroelement interaction using the most physiologically relevant system possible. For instance, here we used CD4-positive human T cells to show that near-physiologic levels of an A3G catalytic mutant (E259Q) failed to inhibit the spreading replication of HIV-1(ΔVif).

ACKNOWLEDGMENTS

We thank L. Mansky for helpful comments, D. Nissley and M. Stenglein for reagents and helpful comments, R. Wright and D. Clark for reagents, Y. Abebe, P. Gross, B. Hoium, J. Strong, and S. Wagner for technical assistance, and the University of Minnesota flow core facility. The A3G and p24 antibodies were obtained from Jaisri Lingappa and Michael Malim through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

This work was supported by NIH grant AI064046. R.S.H. was a Searle Scholar and a University of Minnesota McKnight Land Grant Professor. A.J.S. and D.A.M. were supported in part by Doctoral Dissertation Fellowships from the University of Minnesota Graduate School. G.H. was supported in part by a Canadian Institutes for Health Research doctoral studentship.

ADDENDUM IN PROOF

During the preparation of this manuscript, K. Strebel and colleagues reported a complementary series of experiments indicating that the DNA deaminase activity of APOBEC3G is required for HIV-1 restriction (**E. Miyagi, S. Opi, H. Takeuchi, M. Khan, R. Goila-Gaur, S. Kao, and K. Strebel**, J. Virol. **81:**13346–13353, 2007).

REFERENCES

1. **Bieniasz, P. D.** 2004. Intrinsic immunity: a front-line defense against viral attack. Nat. Immunol. **5:**1109–1115.
2. **Bishop, K. N., R. K. Holmes, and M. H. Malim.** 2006. Antiviral potency of APOBEC proteins does not correlate with cytidine deamination. J. Virol. **80:**8450–8458.
3. **Bogerd, H. P., H. L. Wiegand, A. E. Hulme, J. L. Garcia-Perez, K. S. O'Shea, J. V. Moran, and B. R. Cullen.** 2006. Cellular inhibitors of long interspersed element 1 and Alu retrotransposition. Proc. Natl. Acad. Sci. USA **103:**8780–8785.
4. **Borman, A. M., C. Quillent, P. Charneau, C. Dauguet, and F. Clavel.** 1995. Human immunodeficiency virus type 1 Vif^- mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. J. Virol. **69:**2058–2067.
5. **Chen, K. M., N. Martemyanova, Y. Lu, K. Shindo, H. Matsuo, and R. S. Harris.** 2007. Extensive mutagenesis experiments corroborate a structural model for the DNA deaminase domain of APOBEC3G. FEBS Lett. **581:**4761–4766.
6. **Chiu, Y. L., and W. C. Greene.** 2006. Multifaceted antiviral actions of APOBEC3 cytidine deaminases. Trends Immunol. **27:**291–297.
7. **Chiu, Y. L., V. B. Soros, J. F. Kreisberg, K. Stopak, W. Yonemoto, and W. C. Greene.** 2005. Cellular APOBEC3G restricts HIV-1 infection in resting $CD4^+$ T cells. Nature **435:**108–114.
8. **Courcoul, M., C. Patience, F. Rey, D. Blanc, A. Harmache, J. Sire, R. Vigne, and B. Spire.** 1995. Peripheral blood mononuclear cells produce normal amounts of defective Vif^- human immunodeficiency virus type 1 particles which are restricted for the preretrotranscription steps. J. Virol. **69:**2068–2074.
9. **Cullen, B. R.** 2006. Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. J. Virol. **80:**1067–1076.
10. **Di Noia, J., and M. S. Neuberger.** 2002. Altering the pathway of immunoglobulin hypermutation by inhibiting uracil-DNA glycosylase. Nature **419:**43–48.
11. **Dutko, J. A., A. Schafer, A. E. Kenny, B. R. Cullen, and M. J. Curcio.** 2005. Inhibition of a yeast LTR retrotransposon by human APOBEC3 cytidine deaminases. Curr. Biol. **15:**661–666.
12. **Esnault, C., O. Heidmann, F. Delebecque, M. Dewannieux, D. Ribet, A. J. Hance, T. Heidmann, and O. Schwartz.** 2005. APOBEC3G cytidine deaminase inhibits retrotransposition of endogenous retroviruses. Nature **433:**430–433.
13. **Esnault, C., J. Millet, O. Schwartz, and T. Heidmann.** 2006. Dual inhibitory

effects of APOBEC family proteins on retrotransposition of mammalian endogenous retroviruses. Nucleic Acids Res. **34:**1522–1531.
14. **Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. A. Haseltine, and J. Sodroski.** 1992. Role of *vif* in replication of human immunodeficiency virus type 1 in $CD4^+$ T lymphocytes. J. Virol. **66:**6489–6495.
15. **Gervaix, A., D. West, L. M. Leoni, D. D. Richman, F. Wong-Staal, and J. Corbeil.** 1997. A new reporter cell line to monitor HIV infection and drug susceptibility in vitro. Proc. Natl. Acad. Sci. USA **94:**4653–4658.
16. **Goncalves, J., Y. Korin, J. Zack, and D. Gabuzda.** 1996. Role of Vif in human immunodeficiency virus type 1 reverse transcription. J. Virol. **70:** 8701–8709.
17. **Haché, G., M. T. Liddament, and R. S. Harris.** 2005. The retroviral hypermutation specificity of APOBEC3F and APOBEC3G is governed by the C-terminal DNA cytosine deaminase domain. J. Biol. Chem. **280:**10920–10924.
18. **Harris, R. S., K. N. Bishop, A. M. Sheehy, H. M. Craig, S. K. Petersen-Mahrt, I. N. Watt, M. S. Neuberger, and M. H. Malim.** 2003. DNA deamination mediates innate immunity to retroviral infection. Cell **113:**803–809.
19. **Harris, R. S., D. S. Croom-Carter, A. B. Rickinson, and M. S. Neuberger.** 2001. Epstein-Barr virus and the somatic hypermutation of immunoglobulin genes in Burkitt's lymphoma cells. J. Virol. **75:**10488–10492.
20. **Harris, R. S., and M. T. Liddament.** 2004. Retroviral restriction by APOBEC proteins. Nat. Rev. Immunol. **4:**868–877.
21. **Holmes, R. K., M. H. Malim, and K. N. Bishop.** 2007. APOBEC-mediated viral restriction: not simply editing? Trends Biochem. Sci. **32:**118–128.
22. **Hulme, A. E., H. P. Bogerd, B. R. Cullen, and J. V. Moran.** 2007. Selective inhibition of Alu retrotransposition by APOBEC3G. Gene **390:**199–205.
23. **Iwatani, Y., H. Takeuchi, K. Strebel, and J. G. Levin.** 2006. Biochemical activities of highly purified, catalytically active human APOBEC3G: correlation with antiviral effect. J. Virol. **80:**5992–6002.
24. **Jónsson, S. R., G. Haché, M. D. Stenglein, S. C. Fahrenkrug, V. Andresdottir, and R. S. Harris.** 2006. Evolutionarily conserved and non-conserved retrovirus restriction activities of artiodactyl APOBEC3F proteins. Nucleic Acids Res. **34:**5683–5694.
25. **Kaiser, S. M., and M. Emerman.** 2006. Uracil DNA glycosylase is dispensable for human immunodeficiency virus type 1 replication and does not contribute to the antiviral effects of the cytidine deaminase Apobec3G. J. Virol. **80:**875–882.
26. **Khan, M. A., S. Kao, E. Miyagi, H. Takeuchi, R. Goila-Gaur, S. Opi, C. L. Gipson, T. G. Parslow, H. Ly, and K. Strebel.** 2005. Viral RNA is required for the association of APOBEC3G with human immunodeficiency virus type 1 nucleoprotein complexes. J. Virol. **79:**5870–5874.
27. **Kinomoto, M., T. Kanno, M. Shimura, Y. Ishizaka, A. Kojima, T. Kurata, T. Sata, and K. Tokunaga.** 2007. All APOBEC3 family proteins differentially inhibit LINE-1 retrotransposition. Nucleic Acids Res. **35:**2955–2964.
28. **Kreisberg, J. F., W. Yonemoto, and W. C. Greene.** 2006. Endogenous factors enhance HIV infection of tissue naive CD4 T cells by stimulating high molecular mass APOBEC3G complex formation. J. Exp. Med. **203:**865–870.
29. **Kremer, M., A. Bittner, and B. S. Schnierle.** 2005. Human APOBEC3G incorporation into murine leukemia virus particles. Virology **337:**175–182.
30. **Krokan, H. E., F. Drablos, and G. Slupphaug.** 2002. Uracil in DNA—occurrence, consequences and repair. Oncogene **21:**8935–8948.
31. **Lecossier, D., F. Bouchonnet, F. Clavel, and A. J. Hance.** 2003. Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science **300:**1112.
32. **Liddament, M. T., W. L. Brown, A. J. Schumacher, and R. S. Harris.** 2004. APOBEC3F properties and hypermutation preferences indicate activity against HIV-1 in vivo. Curr. Biol. **14:**1385–1391.
33. **Luo, K., B. Liu, Z. Xiao, Y. Yu, X. Yu, R. Gorelick, and X. F. Yu.** 2004. Amino-terminal region of the human immunodeficiency virus type 1 nucleocapsid is required for human APOBEC3G packaging. J. Virol. **78:**11841–11852.
34. **Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono.** 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature **424:**99–103.
35. **Mariani, R., D. Chen, B. Schrofelbauer, F. Navarro, R. Konig, B. Bollman, C. Munk, H. Nymark-McMahon, and N. R. Landau.** 2003. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell **114:**21–31.
36. **Mbisa, J. L., R. Barr, J. A. Thomas, N. Vandegraaff, I. J. Dorweiler, E. S. Svarovskaia, W. L. Brown, L. M. Mansky, R. J. Gorelick, R. S. Harris, A. Engelman, and V. K. Pathak.** 2007. Human immunodeficiency virus type 1 cDNAs produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration. J. Virol. **81:**7099–7110.
37. **Miyoshi, H., U. Blomer, M. Takahashi, F. H. Gage, and I. M. Verma.** 1998. Development of a self-inactivating lentivirus vector. J. Virol. **72:**8150–8157.
38. **Muckenfuss, H., M. Hamdorf, U. Held, M. Perkovic, J. Lower, K. Cichutek, E. Flory, G. G. Schumann, and C. Munk.** 2006. APOBEC3 proteins inhibit human LINE-1 retrotransposition. J. Biol. Chem. **281:**22161–22172.
39. **Navarro, F., B. Bollman, H. Chen, R. Konig, Q. Yu, K. Chiles, and N. R. Landau.** 2005. Complementary function of the two catalytic domains of APOBEC3G. Virology **333:**374–386.
40. **Newman, E. N., R. K. Holmes, H. M. Craig, K. C. Klein, J. R. Lingappa, M. H. Malim, and A. M. Sheehy.** 2005. Antiviral function of APOBEC3G can be dissociated from cytidine deaminase activity. Curr. Biol. **15:**166–170.
41. **Schäfer, A., H. P. Bogerd, and B. R. Cullen.** 2004. Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor. Virology **328:**163–168.
42. **Schumacher, A. J., D. V. Nissley, and R. S. Harris.** 2005. APOBEC3G hypermutates genomic DNA and inhibits Ty1 retrotransposition in yeast. Proc. Natl. Acad. Sci. USA **102:**9854–9859.
43. **Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646–650.
44. **Shindo, K., A. Takaori-Kondo, M. Kobayashi, A. Abudu, K. Fukunaga, and T. Uchiyama.** 2003. The enzymatic activity of CEM15/Apobec-3G is essential for the regulation of the infectivity of HIV-1 virion but not a sole determinant of its antiviral activity. J. Biol. Chem. **278:**44412–44416.
45. **Simon, J. H., R. A. Fouchier, T. E. Southerling, C. B. Guerra, C. K. Grant, and M. H. Malim.** 1997. The Vif and Gag proteins of human immunodeficiency virus type 1 colocalize in infected human T cells. J. Virol. **71:**5259–5267.
46. **Simon, J. H., T. E. Southerling, J. C. Peterson, B. E. Meyer, and M. H. Malim.** 1995. Complementation of *vif*-defective human immunodeficiency virus type 1 by primate, but not nonprimate, lentivirus *vif* genes. J. Virol. **69:**4166–4172.
47. **Sova, P., and D. J. Volsky.** 1993. Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with *vif*-negative human immunodeficiency virus type 1. J. Virol. **67:**6322–6326.
48. **Stenglein, M. D., and R. S. Harris.** 2006. APOBEC3B and APOBEC3F inhibit L1 retrotransposition by a DNA deamination-independent mechanism. J. Biol. Chem. **281:**16837–16841.
49. **Svarovskaia, E. S., H. Xu, J. L. Mbisa, R. Barr, R. J. Gorelick, A. Ono, E. O. Freed, W. S. Hu, and V. K. Pathak.** 2004. Human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is incorporated into HIV-1 virions through interactions with viral and nonviral RNAs. J. Biol. Chem. **279:**35822–35828.
50. **von Schwedler, U., J. Song, C. Aiken, and D. Trono.** 1993. Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. J. Virol. **67:**4945–4955.
51. **Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen.** 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:**1793–1808.
52. **Wichroski, M. J., G. B. Robb, and T. M. Rana.** 2006. Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. PLoS Pathog. **2:**e41.
53. **Yang, B., K. Chen, C. Zhang, S. Huang, and H. Zhang.** 2007. Virion-associated uracil DNA glycosylase-2 and apurinic/apyrimidinic endonuclease are involved in the degradation of APOBEC3G-edited nascent HIV-1 DNA. J. Biol. Chem. **282:**11667–11675.
54. **Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam, and L. Gao.** 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. Nature **424:**94–98.